EVALUATION OF A NEW HIGH PERFORMANCE LIQUID CHROMATOGRAPHY BASED METHOD FOR VORICONAZOLE ANALYSIS IN BLOOD

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ABSTRACT

Objective: To develop and evaluate a new high-performance liquid chromatography (HPLC) based method for Voriconazole (VRC) analysis in blood.

Study Design: Cross sectional study.

Place and Duration of Study: Department of Chemical Pathology and Endocrinology Armed Forces Institute of Pathology (AFIP), Rawalpindi, from May 2017 to Oct 2017.

Material and Methods: Trough venous blood samples of twenty-five patients on VRC therapy were collected, after five days of starting VRC. Samples were centrifuged. About 100 µl of Lorazepam as internal standard (5.0 µg/ml), was added to 0.5ml of each sample. Then extraction was done with 4.0 ml of mixture of hexane and ethyl acetate. Centrifugation was again done and supernatant layer was collected and dried under nitrogen. It was reconstituted with 200µl of deionized water and acetonitrile. Samples were analyzed on HPLC.

Results: Method was validated in terms of linearity, limit of detection, limit of quantification, precision, accuracy, recovery, analytical specificity and stability. Linearity calibration curve plot for assay obtained was linear over analytical measurement range of 1.0 to 8.0µg/ml and correlation coefficient was 0.99. Mean intra-assay accuracy for 1.0, 5.0 and 8.0 µg/ml was 96%, 98.6%, 99.5% respectively, and percentage Relative Standard Deviation (RSD) was 5.9%, 1.15%, 1.44% respectively. Mean inter-assay accuracy for 1.0, 5.0 and 8.0 µg/ml was 95%, 98.6%, 99.5% respectively, and % RSD was 5.68%, 1.03% and 1.01% respectively. Limit of Detection (LOD) was 0.25 µg/mL and Limit of Quantitation (LOQ) was 1.0 µg/ml.

Conclusion: HPLC based method for VRC determination is accurate and specific with good clinical correlation. This method may be used in routine for Therapeutic Drug Monitoring (TDM) of VRC.

Keywords: High Performance Liquid Chromatography, Therapeutic drug monitoring, Voriconazole.

INTRODUCTION

Invasive fungal infections have become an emerging clinical entity over a past few decades. Most common causative agents include Candida albicans and Aspergillus fumigatus, but other variant fungal species are also emerging, such as non-fumigatus species of aspergillus, opportunistic yeast-like fungi and more than seventeen different species of Candida[1]. Infectious Diseases Society of America (IDSA) guidelines has suggested Voriconazole (VRC) as new and first line treatment option for asper-gillosis, candidiasis, and refractory mycoses[2,3]. Chemical structure of VRC was synthesized from that of fluconazole by replacement of one triazole moiety by a fluoropyrimidine group and alpha methylation. This modification resulted in an enhanced spectrum of antifungal activity and an increased invitro potency[4]. Variability in plasma concentration of VRC, both within and between individuals, is high and this characteristic can be attributed to several factors including nonlinear pharmacokinetics, genetic polymorphism of the cytochrome P450 enzyme CYP2C19, changes in volume of distribution, drug interactions, hepatic dysfunction, and age[5].

Therapeutic Drug Monitoring (TDM) is important to optimize therapy in terms of improving efficacy and reducing toxicity. For TDM of VRC, plasma trough concentration measurement, obtained under steady-state conditions, is preferred and trough concentrations in the range

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Received: 13 Jul 2018; revised received: 01 Nov 2018; accepted: 12 Nov 2018

of 1.5 to 5 µg/ml are important for the prevention of treatment failure and toxicity[6,7]. Its elimination half-life is approximately 6-9 h, and it increases with increasing dosage. Steady-state plasma concentrations are attained after 5–7 days of treatment[8]. Less than 2.0 % of the drug is excreted unchanged in urine; 80% of the drug metabolites are eliminated in urine and remain-der is excreted through fecal route[9].

There are several Liquid Chromatography-Mass Spectrometry (LC–MS) or LC–MS/MS methods available for the quantification of azole agents. As compared to other techniques these are more sensitive and give accurate findings but are more expensive and not available globally. Currently chromatographic apparatus along with ultraviolet (UV) detection are oftenly accessible in clinical laboratories, that are cheap, rapid and easily available, and its management does not require such technical expertise as LC–MS/MS does[10]. Improving therapy in terms of patient toxicity, outcome and survival is contributed by TDM of VRC. The developed fast and simple HPLC with diode-array detection (HPLC-DAD) analysis will enable reliable quantification of VRC. Small sample volume, its simplicity, ease of sample preparation and high throughput of HPLC-DAD method make it particularly suitable for application in a clinical laboratory.

METHODOLOGY

This cross sectional (validation) study was conducted in Department of Chemical Pathology and Endocrinology Armed Forces Institute of Pathology (AFIP), Rawalpindi from May 2017 to October 2017 after approval from Institutional Review Board (IRB). Inclusion criteria included all patients on VRC therapy and whose steady state levels had been achieved, irrespective of age and gender. Patients on VRC therapy, who developed concurrent gastrointestinal symptoms, were excluded from the study. Sampling technique used was non-probability convenient sampling. Twenty-five patients, who were on VRC therapy, were included in the study. Two

Table-I: HPLC parameters for Voriconazole detection in human plasma.

Parameters	Values
Injection volume	20 µl
Wavelength detection	256 nm
Column	Eclipse X DB C18 (4.5×5µm)
Column Temperature	40°C
Mobile phase A	Acetonitrile
Mobile phase B	DH2O
Mobile phase Flow rate	1.0 mL/min
Calibration Mode	Calibration curve
Concentration Units	µg /ml
Run Time	8 minutes
Retention time	2.9 minutes
Post run time	2 minutes

Table-II: Inter assay and intra assay precision and accuracy of HPLC based method for voriconazole.

Level (µg/ml)	Observed (Mean µg/ml ± SD)	Precision (% RSD)	Bias (%)	Accuracy (%)
Inter Assay				
1.0	0.95 ± 0.054	5.68%	5.0%	95%
5.0	4.93 ± 0.051	1.03%	1.4%	98.6%
8.0	7.96 ± 0.081	1.01%	0.5%	99.5%
Intra Assay				
1.0	0.96 ± 0.057	5.9%	4.0 %	96%
5.0	4.93 ± 0.057	1.15%	1.4%	98.6%
8.0	7.96 ± 0.115	1.44%	0.5%	99.5%

(2.0) ml venous blood sample (trough level) was collected from each patient, in EDTA container, after steady state level of drug in patient was achieved i.e. after five days of starting VRC therapy. Samples were transported to laboratory for further analysis. Samples were stored at 4°C, if there was a delay in analysis.

Chemicals and reagents: Standard solutions of the analyte and internal standard were obtained by diluting stock solutions with methanol. Methanolic stock solutions of VRC standard1.0 mg/mL (Sigma Aldrich USA, 5.0 mg) and lorazepam 1.0 mg/ml were used. Lorazepam 5.0 µg/ml was used as internal standard (Cerillient Corporation USA). All solutions were stored protected from light in amber glass bottles. Other reagents used included acetonitrile, methanol, ethyl acetate, n-hexane (99.9%, w/w; Merck Pakistan). For all reagents, that needed reconstitution, HPLC grade ultrapure water (Millipore apparatus, Merck Pakistan) was used.

Sample preparation: Procedure for sample preparation was incorporated by adding 100 µl of internal standard Lorazepam (5 µg/ml) to 0.5ml of sample and then it was extracted with 4.0 ml of hexane and ethyl acetate, in equal quantity. After that, mixture was rotated for 30 minutes and centrifuged for 20 minutes at 4500 revolutions per minute (RPM). Supernatant layer (organic layer) was collected in a separate culture tube, was dried under nitrogen at 60°C and then reconstituted with 200 µl deionized water (DH_2O) and acetonitrile, in equal quantity. Three level quality control samples with known concentrations of 1.0, 4.0, and 7.0µg/mlwere prepared and run during sample analysis of patients.

Assay procedure: Samples were analyzed on Agilent HPLC 1200 Series (table-I) and column Eclipse X DB C18 (4.5×5µm). The injector volume was 20µl and the column was maintained at a temperature of 40°C. Calculations were made against a 5-point calibration curve from serial dilutions of the standard stock solution. Separation was performed using a gradient elution with mobile phase A consisting of acetonitrile, and mobile phase B consisting of DH_2O at a flow-rate of 1.0 ml/min. Two components of the mobile phase were filtered through 0.4µ membrane filters and degassed by an ultrasonic bath for 5 minutes.

Table-III: Analytical specificity (Haemolyzed, lipemic and icteric Sample).

Analytical Specificity	Mean ± SD	% RSD
Haemolysis (3 cycles)	2.95 ± 0.047	1.59%
Lipemia (3 Cycles)	3.01 ± 0.01	0.33%
Icterus(3 Cycles)	3.04 ± 0.011	0.36%

Table-IV: Comparison of international study with our study.

Parameters	Chawla *et al.*	Our study
Mobile phase	Acetonitrile and water (7:3)	Acetonitrile and water (1:1)
Flow rate (ml/min)	1.0	1.0
Wave length (nm)	255	256
Limit of Quantification (LOQ) µg/ml	0.2	1.0
Analytical Measurement Range (AMR) µg/ml	0.2-15	0.5-8
Intra-assay RSD (%)	4.7	5.9
	1.2	1.15
	1.05	1.44
Inter-assay RSD (%)	12.8	5.68
	12.01	1.03
	11.59	1.01
Recovery (%)	117	99
Accuracy (%)	80-120	98

Data Analysis: SPSS version 24 was used for the calculation of descriptive statistics like Mean and SD. Other measures were manually calculated like linearity, recovery, accuracy, precision, limitof detection and quantification, analytical specificity and stability. Linearity calibration curve was made as given by instrument.

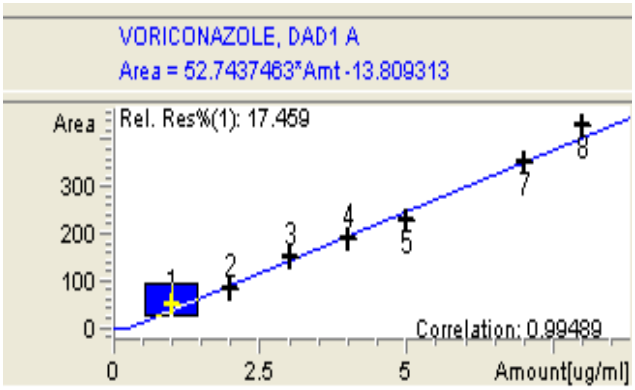

Figure-1: Calibration Curve showing concentration and absorbance (Area).

RESULTS

Validation parameters evaluated during method development in this study are as follows:

Linearity: Linearity calibration curve plot for the assay obtained was linear over the analytical measurement range (AMR) of 1.0 to 8µg/ml, and correlation coefficient was 0.99 (fig-1). Linearity was validated over the same range for seven consecutive days. Result showed significant correlation between area and concentration of analyte.

Limit of detection (LOD) and Limit of quantitation (LOQ): Limit of detection (LOD) was at the level of 0.25 µg/mL and limit of quantification(LOQ) was estimated at the level of 1.0µg/ml.

Accuracy & precision: Accuracy & precision of assay method was evaluated in triplicate at three concentration levels i.e. 1.0, 5.0, and 8.0 µg/mL as given in table-II. Precision was expressed as coefficient of variation whereas accuracy was expressed as percentage of the relative error, determined with the formula [(mean measured concentration/nominal concentration)/nominal concentration] ×100. Acceptance criteria for precision (<15% RSD) and accuracy (bias within ± 15% of the accepted

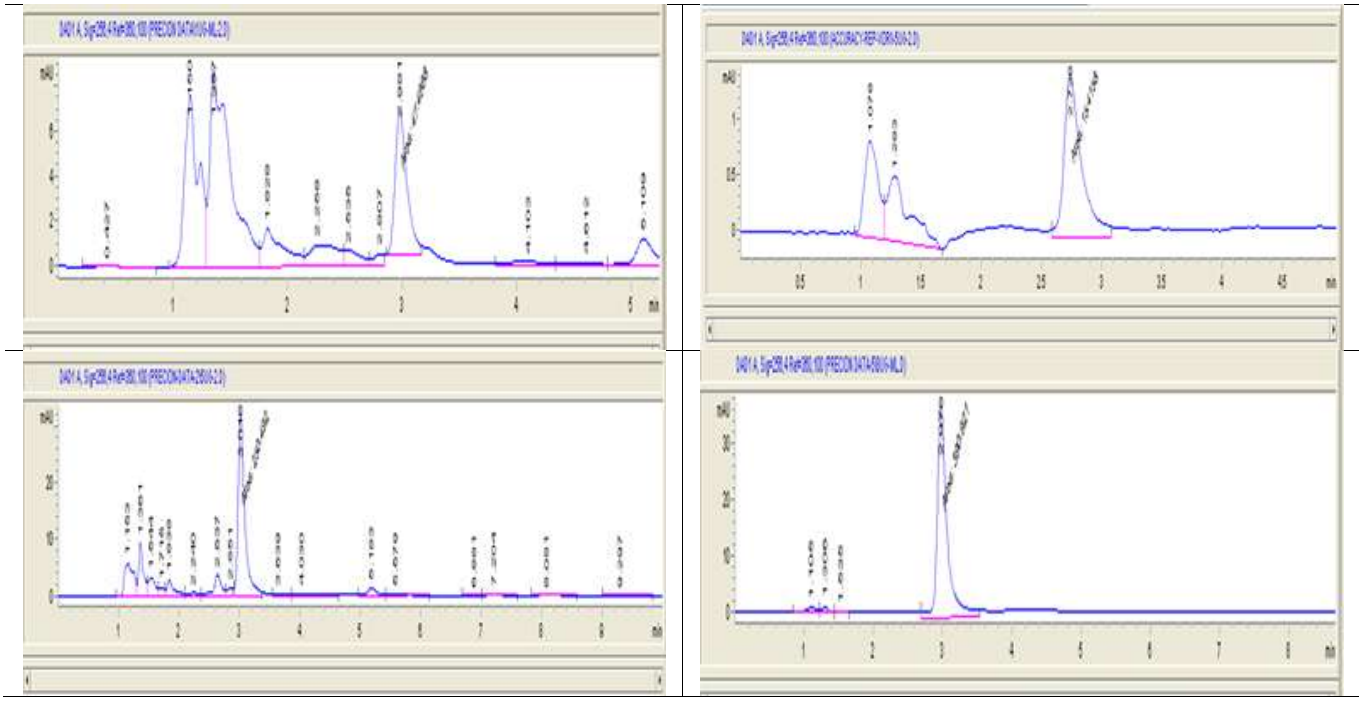

Figure-2: HPLC Chromatograms at different voriconazole concentrations 0.5,1.0,5.0 and 8.0 µg/ml.

reference value) was used, as has been proposed by Shah *et al*[11].

Recovery: Recovery was performed in triplicate with spiked standards at 1.0 µg/ml and 8.0 µg/ml and it showed satisfactory results (98.7% and 99.5% respectively). Percentage RSD was 1.2% and 0.75% respectively.

Analytical specificity: Presence of potentially interfering substances or factors in the sample matrix of haemolyzed, icteric and lipemic sample was measured in triplicate with spiked standard of 3.0 µg/ml and is shown in table-III.

Stability: Sample integrity and stability were checked at different temperatures (30°C and 40°C) and it showed no variability in result. Stability was determined after the exposure of the spiked samples, 1.0, 5.0 and 8.0 µg/ml at room temperature for 4, 24 and 48 hours and the ready-to-inject samples (after extraction) in the HPLC auto sampler at 4°C for 24 h. It shows that this method will be stable at room temperature at 24 and 48 hours for VRC estimation.

DISCUSSION

VRC is a broad-spectrum triazole antifungal agent used in the treatment of a wide range of opportunistic fungal infections, including aspergillosis[4]. Although plasma VRC concentration is unpredictable, TDM could be most useful in order to ascertain clinical efficacy and to minimize toxic effects mainly neurotoxicity and abnormal liver function test results[12]. Different methods exist for detection of VRC in biological fluids, which include HPLC combined with mass spectrometry[13-15]. HPLC with UV detection[10,16.17] and bioassay[18,19]. Bioassay is easier than HPLC methods and needs no special equipment, but its limitation is degree of variations and lack of required sensitivity for VRC therefore, it is not sufficient for pharmacokinetic studies. As every laboratory has neither easy access to LC-MS/MS nor the requisite expertise to handle samples on same, a sensitive, specific and simple HPLC analytical method could be a valuable alternative for determining serum VRC concentrations.

Comparison of international study with this study is given in table-IV. Chawla *et al*[8] used acetonitrile and water in ratio of 7:3, while in this study it was utilized in a ratio of 1:1, as desired sensitivity could only be achieved at this concentration ratio. AMR in comparative study is 0.2-15 µg/ml, while it is 0.5-8 µg/ml in this study. The difference in AMRs can be attributed mostly to method variability and no use of buffer in this method. Upper limit of AMR was lower in this study compared to that by Chawla *et al*[8] but when tested on real patient samples, the range conveniently covered almost all clinical cases on VRC therapy. Intra-assay CV variations were comparable in both studies (table-IV); however, interassay variations were not quite similar; this study thus showing a low CV and hence high inter-assay precision. This lesser variation between assays definitely improves the overall utility of our proposed method for routine clinical monitoring, where a lesser variation is desired among samples received from a single patient at different consecutive days.

A fixed dosing regimen is not optimal for VRC therapy and drug levels would serve as a useful guide for dosing in patients with several influencing factors[8]. By considering the afore-mentioned methodology, findings from the current study illustrated that VRC was stable at most of the implemented conditions. It was recommended to use this methodology for routine TDM in clinical laboratories. In this study, a new method has been developed for voriconazole but there is a need for further evaluation of AMR.

CONCLUSION

HPLC based method for VRC determination is accurate and specific with good clinical correlation. This method may be used in routine for Therapeutic Drug Monitoring (TDM) of VRC.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any authors.

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